Kidney International, Vol. 54 (1998), pp. 1250–1260

Adenovirus-mediated kallikrein gene delivery reverses salt-induced renal injury in Dahl salt-sensitive rats

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Adenovirus-mediated kallikrein gene delivery reverses salt-induced renal injury in Dahl salt-sensitive rats.

Background. The tissue kallikrein-kinin system has been shown to play a role in cardiac and renal functions. In this study, we investigated the ability of kallikrein gene delivery to reverse salt-induced cardiac hypertrophy and renal injury in Dahl salt-sensitive rats.

Methods. Adenovirus harboring the human tissue kallikrein gene, Ad.CMV-cHK, was delivered intravenously into Dahl salt-sensitive rats suffering from hypertension, cardiac hypertrophy and renal damage induced by a high salt diet (4% NaCl) for four weeks.

Results. Expression of human kallikrein mRNA was detected in rat kidney, heart, aorta and liver, and immunoreactive human kallikrein levels were measured in the serum and urine of rats receiving gene delivery. A single injection of Ad.CMV-cHK caused a significant reduction of blood pressure for more than two weeks. Kallikrein gene transfer caused left ventricular mass reduction and elevated glomerular filtration rate, renal blood flow, urinary excretion, urinary kinin, nitrite/nitrate content, cGMP and cAMP levels. Morphological investigations showed that kallikrein gene transfer caused a significant reversal in salt-induced tissue and organ damage. In the heart, cardiac hypertrophy and fibrosis were reduced, and in the kidney, both glomerular sclerotic lesions and tubular damage were reversed.

Conclusions. Adenovirus-mediated kallikrein gene delivery is effective in reversing salt-induced cardiac hypertrophy and renal injury in Dahl-salt sensitive rats.

The tissue kallikrein-kinin system has been shown to play a role in hypertensive and renal diseases [1, 2]. Extensive clinical studies showed that urinary kallikrein levels are inversely correlated with blood pressure [3–5]. Since urinary kallikrein originates from the kidney, the correlation between high blood pressure and reduced urinary kallikrein levels suggests the participation of renal kallikrein in blood pressure homeostasis. Although these clinical studies have implicated a role of renal kallikrein in hypertension, the results are based on random population samples which do not lend themselves to rigorous genetic analyses. A study aimed at identifying genetic factors associated with cardiovascular risks using family pedigrees concluded that a dominant gene expressed as renal or urinary kallikrein may be associated with a reduced risk of hypertension [6]. Reduced urinary kallikrein excretion has also been described in a number of genetically hypertensive rats [7–10]. To evaluate the role of tissue kallikrein in blood pressure regulation as well as cardiovascular and renal function, we have created animal models with altered kallikrein gene expression. We showed that transgenic mice overexpressing human tissue kallikrein exhibit a life-long reduction in blood pressure [11, 12]. These studies provide direct evidence linking alteration of tissue kallikrein gene expression and blood pressure regulation. To further evaluate the potential of kallikrein gene therapy in hypertension, we introduced the human tissue kallikrein gene into various hypertensive rat models by somatic gene delivery [13–16]. A single injection of the human tissue kallikrein gene in the form of naked DNA or in an adenovirus vector induces a prolonged blood pressure reduction in genetically hypertensive or experimentally-induced hypertension for several weeks. These results showed that a continuous supply of exogenous tissue kallikrein via gene delivery exhibits protective effects in the development of high blood pressure.

A continuous supply of kallikrein has also been shown to have protective effects on renal function as long-term infusion of purified tissue kallikrein attenuates glomerular sclerotic lesions and tubular injury in hypertensive Dahl salt-sensitive (Dahl-SS) rats without causing an apparent blood pressure reduction [17]. Renal kallikrein content of Dahl-SS rats was shown to be lower than that of Dahl salt-resistant rats (Dahl-SR) on high, normal, and low salt diets [18]. A previous study showed that renal or urinary kallikrein levels of Dahl-SS rats on a high-salt diet (8% NaCl) were lower than for rats on a normal diet [19]. Lower kallikrein or kinin levels in Dahl-SS rats on a high salt diet may contribute to their high blood pressure and these findings suggest that low renal kallikrein levels may also

Key words: kallikrein, gene delivery, Dahl salt-sensitive rats, renal injury, cardiac hypertrophy, hypertension.

Received for publication January 20, 1998
and in revised form April 22, 1998
Accepted for publication May 11, 1998

contribute to salt-related renal diseases. Our recent studies showed that kallikrein gene delivery offers protection if administered prior to the development of cardiac hypertrophy and renal injury in Dahl-SS rats fed a high salt diet [20]. It is unclear if existing tissue damage can be reversed by this novel technique. In this study, we investigated this possibility and found that kallikrein gene delivery in Dahl-SS rats caused a reversal in cardiac hypertrophy and renal injury induced by a high salt diet prior to gene therapy. These findings provide important information for future therapeutic applications in treating salt-induced end-stage renal diseases.

METHODS

Animal treatment

Dahl salt-sensitive rats (male, four weeks old) were purchased from Sprague-Dawley (Harlan, Indianapolis, IN, USA). Rats were divided into three groups. The control group was fed a standard rat chow (0.4% NaCl) (Harlan Teklad, Madison, WI, USA). The experimental groups were fed throughout the study with a high salt diet (4% NaCl) (Harlan Teklad). All rats had free access to water. Throughout the study period, all animals were housed in a room that was kept at constant temperature (25 ± 1°C) and humidity (60 ± 5%) and was lighted automatically from 8:00 a.m. to 8:00 p.m. All procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Resources, National Academy of Sciences, Bethesda, MD, USA).

Preparation of replication-deficient adenoviral vector, Ad.CMV-cHK

Adenovirus vector Ad.CMV-cHK was prepared as previously described [20] in which the expression of human tissue kallikrein cDNA was under the control of the cytomegalovirus (CMV) enhancer/promoter and was followed by a bovine growth hormone (BGH) poly A signal sequence. Adenovirus harboring the LacZ gene under the control of the CMV enhancer/promoter (Ad.CMV-LacZ) was purchased from the Institute for Human Gene Therapy (Wistar Institute, Philadelphia, PA, USA).

Intravenous delivery of adenoviral vectors Ad.CMV-cHK and Ad.CMV-LacZ

Seven Dahl-SS rats from experimental groups, which were fed a high salt diet containing 4% NaCl, were intravenously (i.v.) injected with either Ad.CMV-cHK or Ad.CMV-LacZ at a dosage of 1.2×10^{10} pfu (plaque formation units) per rat through the tail vein. During the experimental period, blood was collected daily from the tail vein after injection. Rat serum samples were frozen at −80°C until the expression level of immunoreactive human tissue kallikrein could be determined.

Blood pressure measurement

The systolic blood pressure of rats was measured with a manometer-tachometer (Nastume KN-210; Nastume Seisakusho Co. Ltd., Tokyo, Japan) using the tail-cuff method [13]. Unanesthetized rats were introduced into a plastic holder mounted on a thermostatically controlled warm plate, which was maintained at 33 to 35°C during measurements. An average of ten readings was taken for each animal after they became acclimated to the environment.

Urine collection and analysis of physiological parameters

Twenty-four-hour urine of rats was collected in metabolic cages at seven days after gene delivery. Rats were fed a 4% NaCl diet for three hours before placing them in metabolic cages that were supplied with drinking bottles. To eliminate contamination of urine samples, animals received only water during the 24-hour collection period. Urine was collected and centrifuged in a microfuge at 1,000 × g to remove particles. The volume of the supernatant was measured and stored at −20°C until analysis for kinin, nitrite/nitrate (NOx), 3′,5′-cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) and human tissue kallikrein levels.

Tissue preparation

At the end of the experiment, all rats were anesthetized intraperitoneally with pentobarbital at a dose of 50 mg/kg body wt. Blood samples were collected by direct cardiac puncture and chilled at 4°C overnight. The blood samples were centrifuged at 1,000 × g for 20 minutes and sera were removed and frozen at −20°C. At the same time, rats were perfused with normal saline (0.9% NaCl) via the heart. The whole heart, left ventricle, left and right kidneys were removed, blotted and weighed. Tissues of interest were removed and total RNAs were extracted by the trizol method (BRL, Gaitherburg, MO, USA). The extracted RNA was quantified spectrophotometrically by absorbance at 260 nm, dissolved in diethyl pyrocarbonate-treated water, and stored at −80°C for further use.

RT-PCR Southern blot analysis of human tissue kallikrein mRNA

Reverse transcription-polymerase chain reaction (RT-PCR) Southern blot analysis using specific oligonucleotide probes for human tissue kallikrein (5′-primer, 5′-AACACAGCCCAGTTTGT-3′; 3′ primer, 5′-CTTCACATAAGACAGCA-3′; internal probe, 5′-GACCTCAAAATCCTGCC-3′) was performed as previously described [20].

Enzyme-linked immunosorbent assay (ELISA) for human tissue kallikrein

The levels of immunoreactive tissue kallikrein in rat serum and urine were measured by an ELISA specific for

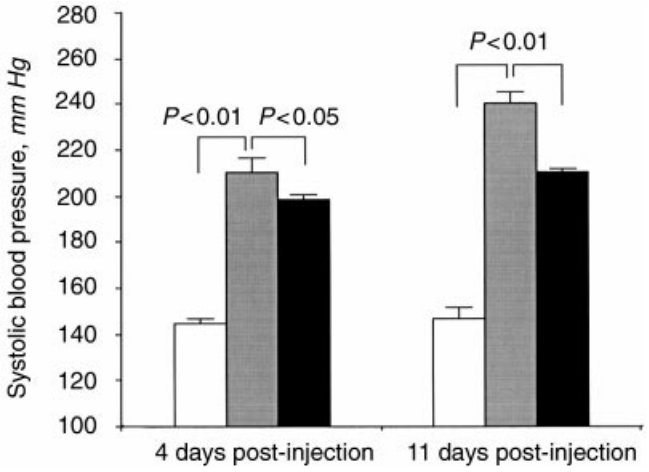


Fig. 1. Systolic blood pressure of Dahl-SS rats after i.v. injection of Ad.CMV-cHK and Ad.CMV-LacZ. Control, Dahl-SS rats (□) were fed a normal salt diet (0.4% NaCl); Dahl-SS rats were fed a high salt diet (4% NaCl), receiving control adenovirus, Ad.CMV-LacZ (▨) or adenovirus carrying the human kallikrein gene, Ad.CMV-cHK (■). Blood pressure values are expressed as mean ± SEM ($N = 6$). Standard errors are shown by bars.

human tissue kallikrein as previously described [20]. Human tissue kallikrein standard ranged from 0.4 to 25 ng/ml. Since the antibody only recognizes active kallikrein [20], the immunoreactive kallikrein levels determined by ELISA represent active kallikrein.

Assays of urinary kinin, cGMP and cAMP levels

Urinary kinin levels were determined by a direct kinin RIA as described [20]. The assays for cGMP and cAMP were conducted according to previously described procedures [21, 22].

Measurement of glomerular filtration rate and renal blood flow

Rats were anesthetized with pentobarbital (50 mg/kg, i.p.) and placed on a heating pad for maintenance of body temperature at 37°C. After tracheotomy, a cannula was placed in the jugular vein for infusion of fluids and drugs. A cannula was placed in the right femoral artery for the measurement of blood pressure and for blood sampling. The bladder was cannulated to allow urine collection from the right kidney. The left kidney was exposed by a flank incision, freed of perirenal tissue, placed in a Lucite cup, and bathed in 0.9% NaCl and then the ureter was cannulated. Hydropenic preparations were maintained by an intravenous injection of 1.2 ml of 0.9% NaCl containing 10% polyfructosan (Inutest, Laevosan, Linz, Austria) and 2% para-aminohippuric acid (PAH; Merck Sharp & Dohme, West Point, PA, USA) via the cannula in the jugular vein during the experimental period. Forty-five minutes were allowed for the preparation to reach a steady state. Timed urine collections were obtained, with blood (0.6 ml) collected between clearance periods. For maintenance of hematocrit, red blood cells from each blood sample were reconstituted to the same volume with 0.9% NaCl and reinjected through the arterial cannula. At the end of each experiment, kidneys were excised, blotted, and weighed. Urine volume was determined gravimetrically. Polyfructosan and PAH concentrations were determined by modified anthrone and colorimetric methods, respectively [23]. Glomerular filtration rate (GFR) and renal plasma flow (RPF) were determined from the clearance of polyfructosan and PAH, respectively. Renal blood flow (RBF) was calculated from RPF and hematocrit. Clearance data were normalized to kidney weight.

Morphological and histological investigation of the heart and kidney

Rats were anesthetized with pentobarbital (50 mg/kg body wt) and hearts and kidneys were removed, cleaned, washed in saline, blotted and weighed. Slides of the kidney and heart were preserved in 4% buffered formaldehyde solution and embedded in paraffin. Five micrometer sections were cut with a microtome, mounted on glass slides and stained with hematoxylin-eosin, then analyzed microscopically and morphometrically. Histological sections of rat heart muscle were analyzed from all experimental groups. Cardiac myocyte diameters were measured in two perpendicular directions using an ocular micrometer with an engraved measuring scale [20]. The ocular micrometer was calibrated against a stage micrometer, and conversion factors were calculated for low (×4 objective) and high (×45 objective) magnifications. Cardiac myocytes were judged to be cut in cross section when the shorter measurement was not more than 2 μm wider than the longer measurement. The average of the two measurements was then recorded as the cross-sectional diameter of the measured myocyte. The mean diameter of 200 cardiomyocytes in each group was measured with a calibrated eyepiece at a magnification of ×450. All sections were evaluated in a blind study in which knowledge of the group to which the measurements belonged was revealed only after the data were tabulated.

Statistical analysis

Data were analyzed using standard statistical methods. Repeated blood pressure measurements at each time point were taken for comparison between control and experimental groups. The blood pressure data were analyzed with the use of ANOVA and Fisher's protected least significant differences. Group data are expressed as mean ± SEM. Values were considered significantly different at a value of $P < 0.05$.

RESULTS

Kallikrein gene delivery reduces blood pressure in hypertensive Dahl-SS rats on a high salt diet

Dahl-SS rats (four weeks old) were fed a high salt (4% NaCl) diet or normal control diet (rat chow, 0.4% NaCl)

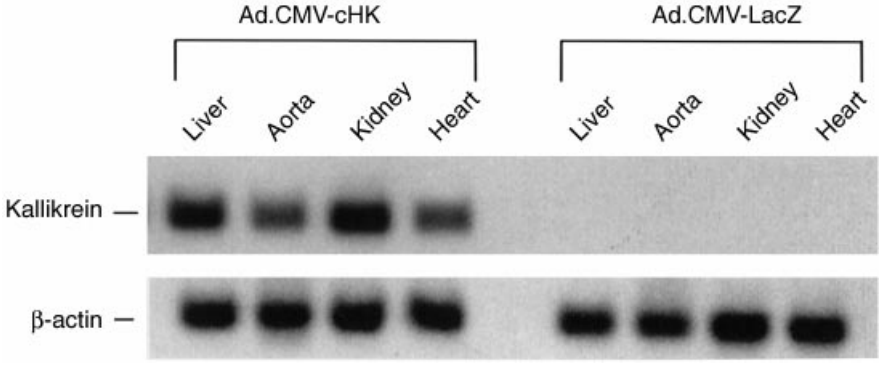


Fig. 2. Expression of human kallikrein mRNA in Dahl-SS rats after human kallikrein gene delivery. The rats were sacrificed at 12 days post injection and one μg of RNA was used for RT-PCR. Human kallikrein mRNA in rat tissues was amplified by a set of specific oligonucleotides for human tissue kallikrein which yielded a partial cDNA (503 bp) product as shown in the upper panel. Rat β-actin mRNA in rat tissues was amplified by a set of specific oligonucleotides which yielded a 500 bp product as shown in the lower panel. RNAs from heart, kidney, aorta and liver of rats receiving Ad.CMV-cHK of Ad.CMV-LacZ are indicated in the Figure.

Table 1. Physiological analysis of Dahl salt-sensitive (SS) rats after kallikrein gene delivery

Variables	(1) Control (0.4% NaCl)	(2) Ad.CMV-LacZ (4% NaCl)	(3) Ad.CMV-cHK (4% NaCl)
Urine volume *ml/100 g body wt/day*	7.4 ± 1.0	9.2 ± 1.5	13.7 ± 0.7[a]
Urinary kinin *ng/100 g body wt/day*	5.4 ± 0.9	12.0 ± 4.4	35.4 ± 9.0[a]
Urinary cGMP *nmol/100 g body wt/day*	11.5 ± 2.9	11.4 ± 2.0	17.6 ± 1.9[a]
Urinary cAMP *nmol/100 g body wt/day*	12.9 ± 1.0	15.1 ± 0.9	18.5 ± 1.0[a]
Urinary NOx content *μmol/100 g body wt/day*	1.45 ± 0.53[b]	0.13 ± 0.07	0.83 ± 0.27[a]
Human kallikrein in serum *ng/ml*	ND	ND	254.1 ± 0.9
Human kallikrein in urine *μg/100 g body wt/day*	ND	ND	16.3 ± 3.6

Dahl-SS rats received either an Ad.CMV-cHK or Ad.CMV-LacZ injection at eight weeks of age and physiological measurements of rats were performed seven days post gene delivery. Six rats from each group were measured for urine excretion, urinary kinin, cGMP, cAMP, NOx content, and human kallikrein levels. Serum and urine samples were collected at 3 and 7 days, respectively, post gene delivery. Data were analyzed with ANOVA and Fisher's protected least significant differences. Values for each group are reported as mean ± SEM (n = 6). A value of $P < 0.05$ was interpreted as indicating a significant difference between the groups. ND means not detectable.

[a] $P < 0.05$, (3) vs. (1) and (2)

[b] $P < 0.01$, (1) vs. (2)

for four weeks. The blood pressure of Dahl-SS rats on a 4% NaCl diet increased with the change in dietary salt intake and the differences between low and high salt groups reached over 50 mm Hg prior to kallikrein gene delivery. Rats (eight weeks old) on a high salt diet were then divided into two groups and injected via the tail vein with either a viral vector containing the human tissue kallikrein gene (Ad.CMV-cHK) or a control vector containing the bacterial LacZ gene (Ad.CMV-LacZ). The blood pressure of Dahl-SS rats fed a high salt diet increased markedly as compared to rats on a low salt diet (0.4% NaCl). Figure 1 shows systolic blood pressures of Dahl-SS rats fed a high salt diet injected with the virus carrying the kallikrein gene or control adenovirus. Delivery of the human tissue kallikrein gene resulted in a significant reduction of blood pressure in salt-induced hypertensive Dahl-SS rats at 4 and 11 days post-injection. The difference in blood pressure between the control group and the group receiving kallikrein gene delivery persisted for more than two weeks post-injection.

Expression of human tissue kallikrein after gene delivery

Expression of the human tissue kallikrein mRNA in Dahl-SS rats after gene delivery was detected by RT-PCR followed by Southern blot analysis using three oligonucleotides specific for human tissue kallikrein. Total RNAs were prepared from tissues of rats 12 days after gene delivery. Figure 2 shows that human kallikrein mRNA can be detected in the kidney, heart, aorta and liver (upper panel, left). The RT-PCR products from rats receiving the Ad.CMV-LacZ gene did not hybridize to the human tissue kallikrein gene probe (upper panel, right). Similar levels of β-actin mRNA were detected in tissues of both experimental and control groups, verifying the quality of RNA in these samples (lower panel). These results indicate that Southern blot analysis is specific for human tissue kallikrein and that endogenous rat tissue kallikrein family members do not interfere with the assay.

Following intravenous injection of Ad.CMV-cHK adenovirus, human tissue kallikrein levels in rat sera and urine, collected at different time periods, were measured by ELISA. The highest level of immunoreactive human tissue kallikrein in rat serum was 254.1 ± 0.9 ng/ml on the third day after gene delivery (Table 1). Also, immunoreactive human tissue kallikrein was measured in the urine of Dahl-SS rats receiving Ad.CMV-cHK (16.3 ± 3.6 μg/100 g body wt/day) but not in the urine of control rats receiving Ad.CMV-LacZ (Table 1). Linear displacement curves for immunoreactive kallikrein in rat sera and urine were

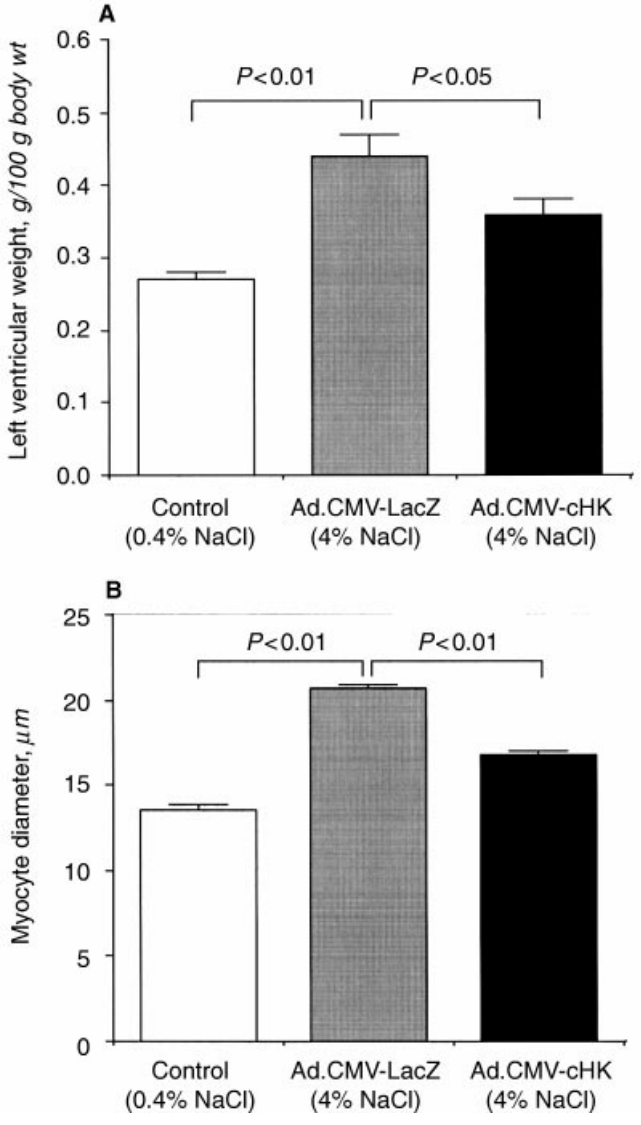


Fig. 3. Left ventricular weight (*A*) and cardiomyocyte diameter (*B*) of Dahl-SS rats after adenovirus-mediated kallikrein gene delivery. Control, on a normal salt diet (0.4% NaCl); Ad.CMV-LacZ, (4% NaCl) receiving control adenovirus carrying the LacZ gene; Ad.CMV-cHK, (4% NaCl) receiving adenovirus carrying the human kallikrein gene.

parallel with the standard curve of human tissue kallikrein, indicating their immunological identity (data not shown).

Increased urinary excretion, kinin, nitrite\nitrate, cAMP, cGMP and human tissue kallikrein levels in rats receiving kallikrein gene delivery

Table 1 shows urinary excretion, kinin, nitrite/nitrate (NOx) content, cAMP and cGMP levels in Dahl-SS rats at seven days after gene delivery. Urine volume significantly increased in rats receiving kallikrein gene delivery as compared to control rats (13.7 ± 0.7 vs. 9.2 ± 1.5 ml/100 g body wt per day, mean ± SEM; $N = 6$, $P < 0.05$). Urinary kinin levels increased by three-fold after kallikrein gene delivery as compared to control rats receiving Ad.CMV-LacZ (35.4 ± 9.0 vs. 12.0 ± 4.4 ng/100 g body wt/day, $N = 5$, $P < 0.01$). Urinary cAMP content increased significantly after kallikrein gene delivery as compared to control rats receiving Ad.CMV-LacZ (18.5 ± 1.0 vs. 15.1 ± 0.9 nmol/100 g body wt/day, $N = 5$, $P < 0.05$). Urinary cGMP levels increased by 1.5-fold after kallikrein gene delivery as compared to control rats receiving Ad.CMV-LacZ (17.6 ± 1.9 vs. 11.4 ± 2.0 nmol/100 g body wt/day, $N = 5$, $P < 0.01$). The urinary NOx content increase-d significantly after kallikrein gene delivery as compared to control rats receiving the LacZ gene (0.83 ± 0.27 vs. 0.13 ± 0.07 μmol/100 g body wt/day, $N = 4$, $P < 0.05$).

Morphological changes in the heart after gene delivery

Figure 3A shows that the left ventricular weight is significantly increased in Dahl-SS rats on a high salt diet (4% NaCl) and injected with Ad.CMV-LacZ, as compared to control rats given a normal salt diet (0.4% NaCl; 0.44 ± 0.03 vs. 0.27 ± 0.01 g per 100 g body wt, mean ± SEM; $N = 6$, $P < 0.05$). The left ventricular weight is significantly decreased in Dahl-SS rats given 4% NaCl in the diet, but injected with the tissue kallikrein gene (Ad.CMV-cHK) as compared to rats injected with Ad.CMV-LacZ (0.38 ± 0.04 vs. 0.44 ± 0.03 g per 100 g body wt, mean ± SEM; $N = 6$, $P < 0.05$). The high salt loading resulted in an enlarged average diameter of cardiomyocytes in Dahl-SS rats with Ad.CMV-LacZ injection (Fig. 3B). Figure 3B shows that the average diameter of cardiomyocytes in the group receiving kallikrein gene transfer is significantly less than that of the Ad.CMV-LacZ group (16.8 ± 0.2 vs. 20.7 ± 0.2 μm, mean ± SEM; $N = 200$, $P < 0.01$). Cardiac myocytes of animals on a normal salt diet (0.4% NaCl) appeared normal and uniform in diameter (13.6 ± 0.3 μm, mean ± SEM; $N = 200$; Fig. 4A), and were used as the baseline of comparison for the high salt and gene-injected animals. Diffuse interstitial proliferation was occasionally found in the LacZ group (Fig. 4C) but not in the group receiving kallikrein gene delivery (Fig. 4D). These results indicate that salt-induced cardiac hypertrophy can be at least partially reversed by kallikrein gene delivery in Dahl-SS rats.

Effects of kallikrein gene delivery on renal function

Glomerular filtration rate in Dahl-SS rats fed a high salt diet and injected with control adenovirus Ad.CMV-LacZ decreased by 35% compared to control rats on a normal salt diet (0.31 ± 0.13 vs. 0.91 ± 0.08 ml/min/g kidney weight, mean ± SEM, $N = 4$, $P < 0.05$). After kallikrein gene delivery, glomerular filtration rate in Ad.CMV-cHK-injected rats increased 2.1-fold as compared to those rats injected with Ad.CMV-LacZ (0.66 ± 0.07 vs. 0.31 ± 0.13 ml/min/g kidney weight, mean ± SEM, $N = 4$, $P < 0.05$; Fig. 5). Similarly, renal blood flow in Dahl-SS rats fed a high salt diet and injected with control adenovirus Ad.CMV-LacZ was reduced 56% compared to control rats on a normal salt diet (6.3 ± 1.9 vs. 11.8 ± 2.8 ml/min/g kidney weight,

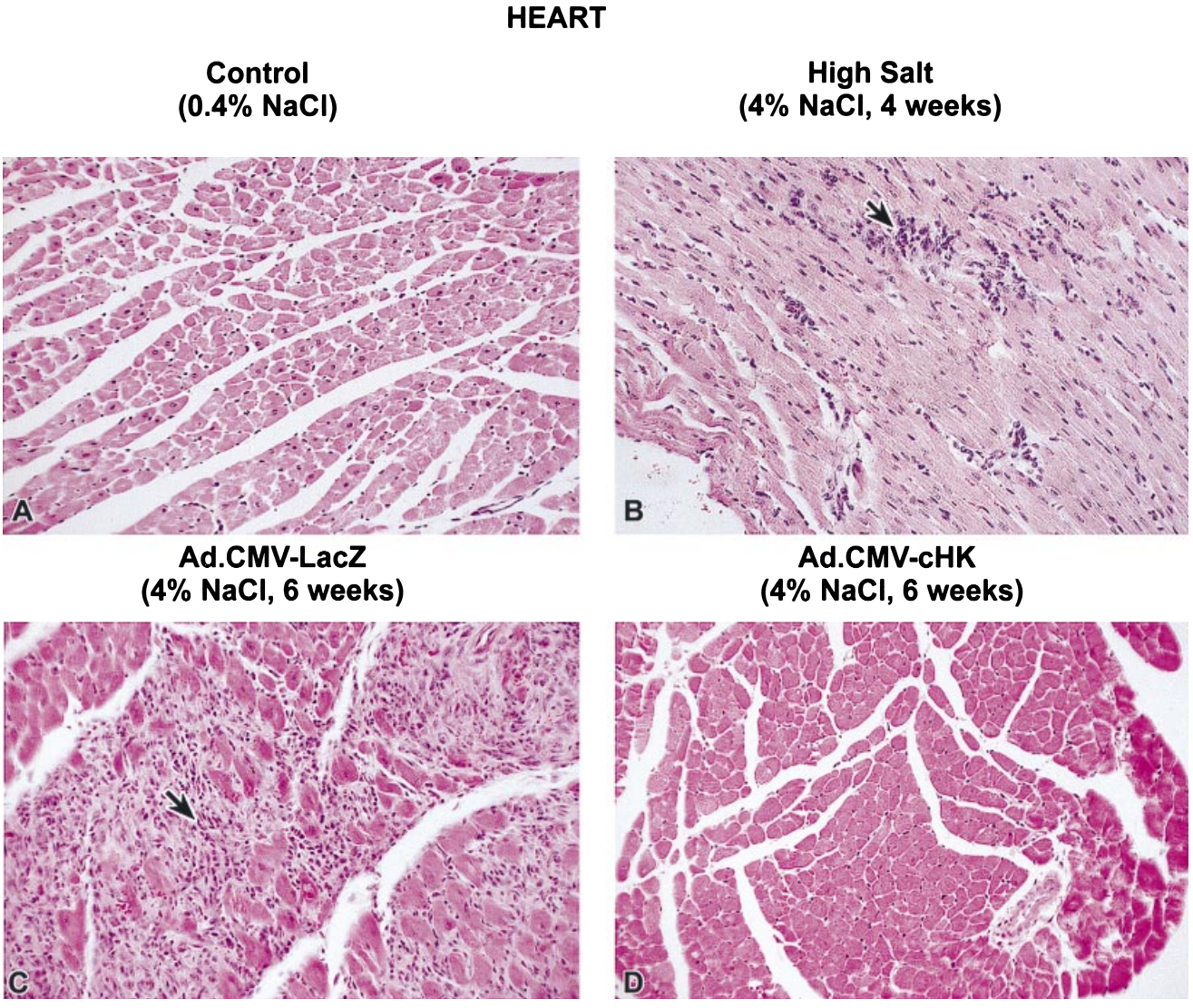


Fig. 4. Histological sections of heart stained with hematoxylin and eosin. (*A*) Control, Dahl-SS rat fed a normal salt diet (0.4% NaCl). (*B*) Dahl-SS rat fed a high salt diet (4% NaCl) for four weeks. (*C*) Dahl-SS rat fed a high salt diet (4% NaCl) for four weeks, then given control adenovirus, Ad.CMV-LacZ, two weeks prior to sacrifice. (*D*) Dahl-SS rat fed a high salt diet (4% NaCl) for four weeks, then given adenovirus, Ad.CMV-cHK carrying the human tissue kallikrein gene, two weeks prior to sacrifice. Interstitial proliferation among enlarged cardiomyocytes is indicated by an arrow (magnification ×125).

mean ± SEM, $N = 4$, $P < 0.05$) while kallikrein gene delivery caused a 1.9-fold increase as compared to those rats injected with Ad.CMV-LacZ (12.0 ± 2.0 vs. 6.3 ± 1.9 ml/min/g kidney weight, mean ± SEM, $N = 4$). These results suggest that salt-induced renal damage leads to impairment of glomerular filtration and renal blood flow, and that kallikrein gene delivery causes reversal of renal damage.

Morphological changes in the kidney after gene delivery

Histological sections of the renal cortex (Fig. 6) and medulla (Fig. 7), stained with hematoxylin and eosin, showed some reversal of salt-induced renal injury in Dahl-SS rats after kallikrein gene delivery. The cortex and medulla of control Dahl-SS rats fed a normal salt diet generally appeared normal, although small casts were occasionally seen in medullary tubules (Figs. 6A and 7A). High salt loading for four weeks resulted in significant renal injury in both the cortex (Fig. 6) and the medulla (Fig. 7). In the cortex of animals treated with either high salt alone (four weeks) or high salt (six weeks) plus Ad.CMV-LacZ for two weeks, damage was marked. This included decreased cell height and loss of brush borders in proximal tubules (Fig. 6 B, C), thickening of glomerular basement membranes with apparent glomerular sclerosis (Fig. 6C), and focal accumulation of inflammatory cells. Sites of focal (micro) hemorrhage were observed in Dahl-SS rats fed a

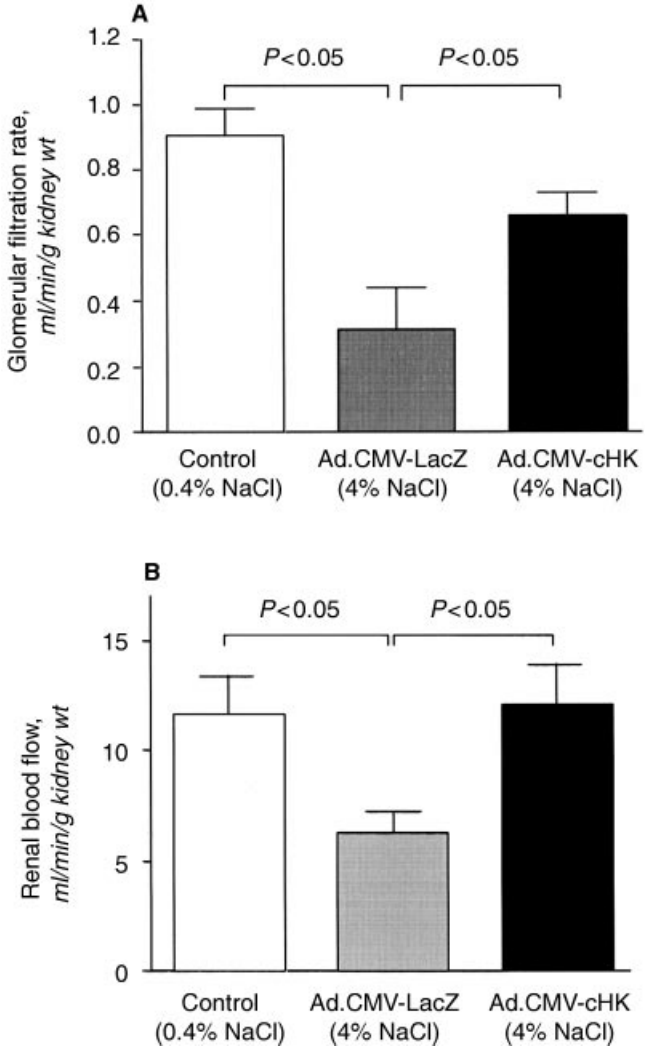


Fig. 5. Glomerular filtration rate (*A*) and renal blood flow (*B*) of Dahl-SS rats after adenovirus-mediated kallikrein gene delivery. Control, on a normal salt diet (0.4% NaCl); Ad.CMV-LacZ, (4% NaCl) receiving control adenovirus carrying the LacZ gene; Ad.CMV-cHK, (4% NaCl) receiving adenovirus carrying the human kallikrein gene. The renal functional study was performed at two weeks after gene delivery.

high salt diet with and without Ad.CMV-LacZ for two weeks (data not shown). Thickening of the arterial muscular layer (media) was obvious in both arcuate and interlobular arteries in these animals. In Dahl-SS fed a high salt diet and receiving kallikrein gene delivery (Ad.CMV-cHK), proximal tubules and glomeruli exhibited much less damage (Fig. 6D). No examples of hemorrhage were observed, and inflammatory cell infiltration appeared decreased. The arterial media was, in most cases, intermediate in thickness between low-salt animals and high-salt animals receiving Ad.CMV-LacZ for two weeks. In addition, it is intriguing that mitoses were often seen in proximal tubule cells, and occasionally in collecting duct cells in animals receiving kallikrein gene delivery (Ad.CMV-cHK) (data not shown).

Figure 8 shows the protective effect of salt-induced glomerular sclerosis in Dahl-SS rats after kallikrein gene delivery. Of 75 glomeruli counted in control rats on a low salt diet, only 1.0 ± 1.3% exhibited sclerosis, compared with 25.8 ± 4.5% of 90 glomeruli counted in LacZ animals fed a high salt diet for six weeks ($P < 0.01$). Of 70 glomeruli counted in Dahl-SS rats receiving kallikrein gene therapy and a high salt diet, 12.1 ± 3.8% exhibited sclerotic changes which is a 50% reduction of glomerular damage, compared to the LacZ group ($P < 0.05$).

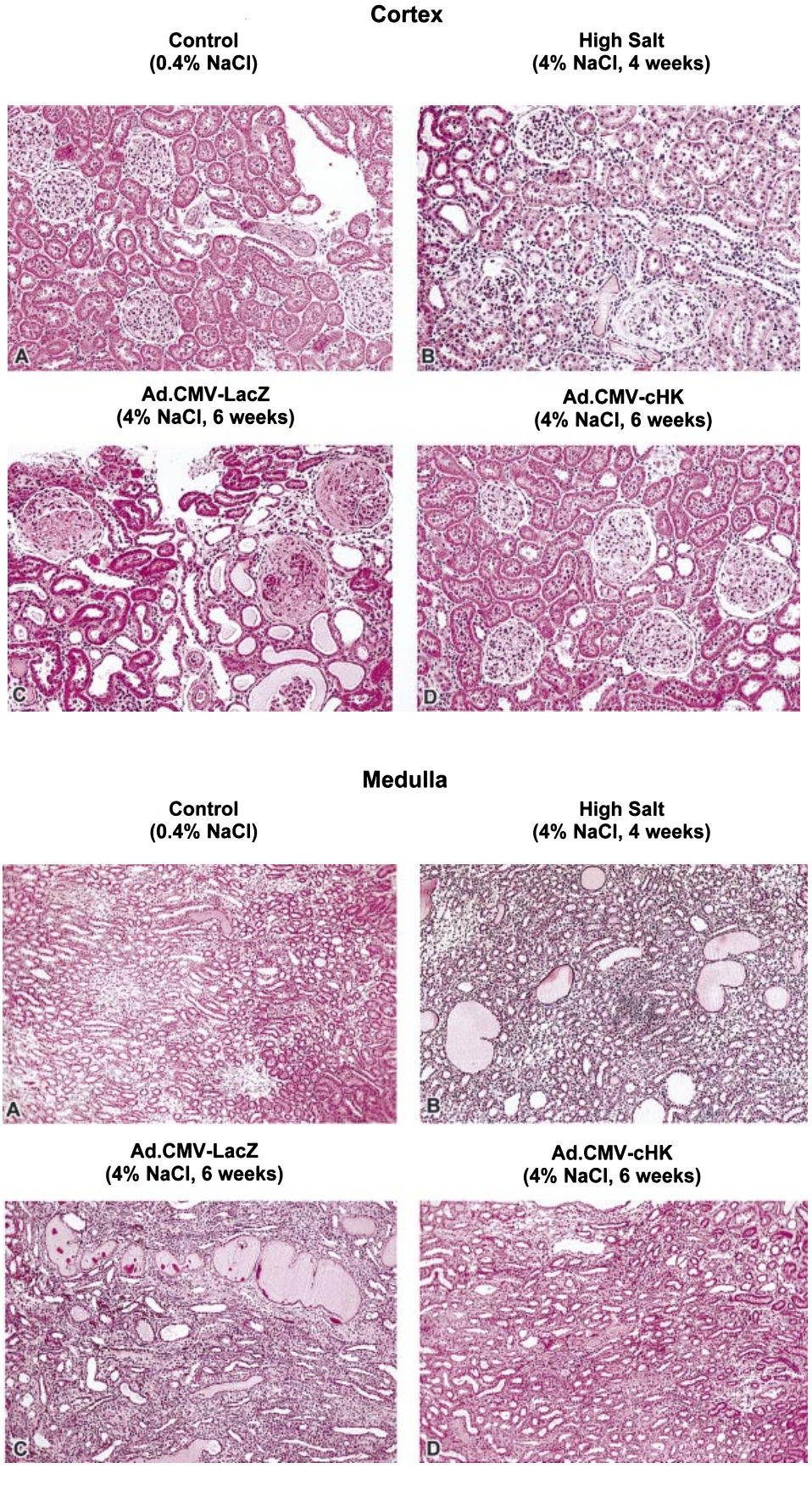
In the medulla (Fig. 7), salt loading resulted in the development of large colloidal casts in renal tubules (compare Fig. 7 A and B). These were also present in animals receiving the control LacZ gene (Ad.CMV-LacZ; Fig. 7C), but greatly reduced in rats receiving kallikrein gene delivery (Ad.CMV-cHK; Fig. 7D). These results indicate that adenovirus-mediated kallikrein gene delivery improved salt-induced renal dysfunction and partially reversed morphological evidence of injury in Dahl-SS rats.

DISCUSSION

The present study shows that a continuous supply of tissue kallikrein by somatic gene delivery reverses salt-induced cardiac hypertrophy and renal injury in Dahl-SS rats. A single injection of the adenovirus carrying the human tissue kallikrein gene resulted in prolonged expression of exogenous kallikrein and increased kinin production. In our previous study, we showed that blood pressure reduction following kallikrein gene delivery was abolished by aprotinin, a potent tissue kallikrein inhibitor, and by Hoe 140, a specific bradykinin B_2 receptor antagonist [13, 14]. These findings indicated that the protective effect following kallikrein delivery may be mediated by activation

Fig. 6. Photomicrographs of representative hematoxylin and eosin-stained renal cortex sections. (*A*) Control, Dahl-SS rat, fed on a normal salt diet (0.4% NaCl). (*B*) Dahl-SS rat, fed a high salt diet (4% NaCl) for four weeks. (*C*) Dahl-SS rat fed a high salt diet (4% NaCl) for four weeks, then given control adenovirus, Ad.CMV-LacZ two weeks prior to sacrifice. (*D*) Dahl-SS rat fed a high salt diet (4% NaCl) for four weeks, then given adenovirus, Ad.CMV-cHK carrying the human tissue kallikrein gene two weeks prior to sacrifice. Note dilated proximal tubules in B and C and glomerular sclerosis in C (magnification ×100).

Fig. 7. Photomicrographs of representative hematoxylin and eosin-stained renal medulla sections. (*A*) Control, Dahl-SS rat fed a normal salt diet (0.4% NaCl). (*B*) Dahl-SS rat, fed on a high salt diet (4% NaCl) for four weeks. (*C*) Dahl-SS rat fed a high salt diet (4% NaCl) for four weeks, then given control adenovirus, Ad.CMV-LacZ, two weeks prior to sacrifice. (*D*) Dahl-SS rat fed a high salt diet (4% NaCl) for four weeks, then given adenovirus, Ad.CMV-cHK carrying the human tissue kallikrein gene, two weeks prior to sacrifice. Note the large colloidal casts in medullary tubules of animals given high salt (Fig. 7B) and high salt plus the Lac Z gene (Fig. 7C) (Magnification ×50).


Cortex
Control
(0.4% NaCl)
High Salt
(4% NaCl, 4 weeks)
A
B
Ad.CMV-LacZ
(4% NaCl, 6 weeks)
Ad.CMV-cHK
(4% NaCl, 6 weeks)
C
D
Medulla
Control
(0.4% NaCl)
High Salt
(4% NaCl, 4 weeks)
A
B
Ad.CMV-LacZ
(4% NaCl, 6 weeks)
Ad.CMV-cHK
(4% NaCl, 6 weeks)
C
D

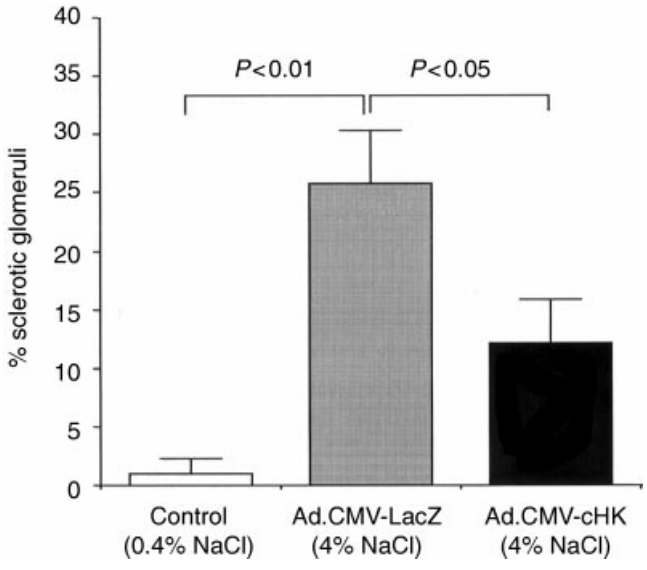


Fig. 8. Protective effect against salt-induced glomerular sclerosis in Dahl-SS rats after kallikrein gene delivery. Control, on a normal salt diet (0.4% NaCl); Ad.CMV-LacZ, (4% NaCl) receiving control adenovirus carrying the LacZ gene; Ad.CMV-cHK, (4% NaCl) receiving adenovirus carrying the human kallikrein gene.

of the kinin system. Increased urinary or renal kinin levels are accompanied by increases in urinary NOx content, cGMP and cAMP levels. The results suggest that the protective effects of kinins on cardiac and renal functions are mediated by the release of NOx and/or eicosanoids resulting in increased cGMP and cAMP levels.

Urinary kallikrein excretion is markedly reduced in salt-sensitive rats as compared with their salt-resistant counterparts [24, 25]. A previous study from this laboratory showed that high NaCl intake suppresses the expression of the kallikrein-kinin system in Dahl-SS rats [19]. When Dahl-SS rats are placed on a high salt diet early in life, they develop hypertension, cardiac hypertrophy and fibrosis as well as renal injury, and die after six to eight weeks. In this study, we placed four-week-old Dahl-SS rats on a high salt diet for four weeks and showed that the blood pressure of these animals reached a level that was 50 mm Hg higher than those on a low salt diet. The high salt diet also resulted in enlarged cardiomyocyte diameter, fibrosis and renal damage in both cortex and medulla. The renal lesions induced by high salt diet are of a focal nature and comparable to malignant hypertensive renal disease seen in humans [26–28]. Histological examinations revealed glomerular basement membrane thickening, renal tubular dilation and disruption of the proximal tubular brush border and protein casts in tubules. To rule out adenovirus as the cause of these lesions, we carried out parallel studies using saline injection to show that similar blood pressure rise and histological lesions were produced in Dahl-SS rats by high salt loading without virus (unpublished results). The genetic basis for salt sensitivity in Dahl-SS rats is not known. In the present study, we showed that a continuous supply of exogenous kallikrein via gene delivery could regulate water, electrolyte transport and salt handling and compensate for the deficiency of the system in Dahl-SS rats after salt loading.

It has been long suspected that enhanced kallikrein-kinin function may alleviate some of the salt-induced damage in Dahl-SS rats. The kallikrein-kinin system is well known for its ability to cause vasodilation, which should provide direct relief for salt-induced hypertension. Kinin has been shown to be responsible for, at least in part, the cardioprotective effect of angiotensin converting enzyme (ACE) inhibitors on myocardial ischemia/reperfusion injury, since the cardiac protective effects of ACE inhibition can be reversed by Hoe140, a bradykinin B2 receptor antagonist [29–32]. Moreover, kinin is known to play a significant role in renal sodium excretion and thus may be particularly relevant in Dahl-SS rats under salt loading. A previous study showed that infusion of purified rat tissue kallikrein via minipumps attenutes renal injury in Dahl-SS rats after salt loading [17]. Collectively, these findings suggest that kallikrein's protective effect in salt-induced cardiac and renal lesions may be mediated by kinin. Taken together, it seems reasonable that elevated activity of the kallikrein-kinin system may offer cardiac protection and improve renal electrolyte handling in this salt sensitive animal model. One possible way to achieve this is by inhibiting kinin's degradation enzyme, kininase II, also known as ACE, using ACE inhibitors. Alternatively, it has been shown that infusion of purified rat tissue kallikrein resulted in attenuation of glomerular sclerotic lesions and tubular injury in Dahl-SS rats on a high salt diet [17]. Based on these observations, we demonstrated that tissue kallikrein levels can be maintained on a long-term basis by somatic gene delivery *in vivo* [13]. This technique was used to deliver the kallikrein gene to Dahl-SS rats and shown to attenuate salt-induced renal injury as well as cardiac hypertrophy if administered prior to organ damage [20].

Our study was undertaken to determine if kallikrein gene delivery can exert beneficial effects in animals with established cardiac hypertrophy and renal injury. To accomplish this aim, we delivered the kallikrein gene in an adenovirus vector, Ad.CMV-cHK, into Dahl rats with existing cardiovascular and renal damage induced after four weeks of salt loading. We demonstrated that kallikrein gene delivery was able to alleviate cardiac hypertrophy and fibrosis, as there was a reduction of cardiomyocyte diameter and left ventricular weight. Most interestingly, we observed a reversal of salt-induced glomerulosclerosis and proximal tubular damage consistent with increases in glomerular filtration rate and renal blood flow. In addition, human tissue kallikrein gene expression in Dahl-SS rats also attenuated salt-induced enlargement of renal mass [20]. It has been shown that losartan is capable of reducing the rise in blood

pressure and glomerular sclerosis index in SHR that are subjected to five-sixths nephrectomy, and that it decreases interstitial fibrosis in cyclosporine-treated Sprague-Dawley rats [33, 34]. These results indicated that losartan can decrease blood pressures as well as ameliorate the organ structural and functional changes. However, infusion of purified kallikrein via minipumps was shown to attenuate salt-induced glomerular sclerosis without affecting the time-dependent elevation of blood pressure in Dahl-SS rats [17]. Further studies in comparing the blood pressure-lowering and renal protective effects of kallikrein gene delivery versus hydralazine/furosemide should clarify the question of whether the improvement in renal structure and function following kallikrein gene delivery is a direct effect of kinin action or a secondary effect due to blood pressure reduction. Our present studies show that kallikrein gene delivery not only has a protective effect on salt-induced cardiac and renal injury, but also could reverse the damage that was already produced by a high salt loading.

The mechanism by which exogenous tissue kallikrein expression can prevent and even reverse salt-induced renal injury is not clear at the present time. One potential function of kallikrein is its ability to process growth factors [35]. A number of peptide growth factors, such as platelet-derived growth factor, fibroblast growth factor, transforming growth factor-α, and epidermal growth factor, are known to elicit mesangial cell proliferation *in vitro* [36]. Kallikrein may thus promote the regeneration of damaged renal cells by accelerating the processes of growth factors. Alternatively, elevated kinin levels caused by increased production of kallikrein may be responsible for the observed reversal of renal damage. Kinin's actions are mediated by B_2 receptors that are widely distributed in the kidney [37]. Kinin is known to stimulate the proliferation of lymphocytes, fibroblasts and arterial smooth muscle cells as well as mesangial cells [38–41]. The mitogenic activity of kinin is abolished by a specific bradykinin receptor antagonist, Hoe140, indicating the involvement of the bradykinin B_2 receptor in mitogenic signal transduction [41]. Current evidence suggests that kinin may play a role in the regulation of DNA synthesis during later stages of nephrogenesis and modulate segmental nephron maturation [41, 42]. The functional significance of kinin's ability to stimulate DNA synthesis and cell proliferation has not been investigated in the kidney of adult animals. In a preliminary histological study, we have observed mitotic figures in proximal tubular cells and occasionally in collecting ducts of rats receiving kallikrein gene delivery in both salt-induced and drug-induced renal injury following kallikrein gene delivery [43]. It is therefore likely that kinin-induced tubular cell proliferation and regeneration may be responsible for the partial reversal of renal damage. Further studies are needed to verify this hypothesis. Also unresolved at this time is the degree to which kallikrein gene delivery can reverse the renal damage. We suspect that there is a point in renal disease progression beyond which reversal is not possible.

Kallikrein gene delivery may be a good experimental candidate in the treatment of salt-related hypertension as well as cardiovascular and renal diseases. Currently, adenovirus-mediated gene delivery can achieve a high level of expression but with limited duration, due mainly to immunosurveillance by the host [44]. Future development of adenovirus gene transfer combined with the administration of immunosuppressive drugs or gene delivery with adeno-associated viruses may offer alternatives for long-term and high-efficiency therapeutic applications.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants HL29397 and HL56686. We thank the Vector Core (Institute of Human Gene Therapy) for providing the adenoviral shuttle plasmid pAdLink.1, and the Wistar Institute, Institute of Human Gene Therapy, for providing adenovirus Ad.CMV-LacZ and Ad.CMV-cHK. We thank Dr. Thomas H. Hintze at New York Medical College for help in the measurement of NOx content and Dr. Jo Anne Simson for critical reading of the manuscript and for evaluation of histological changes in the kidney and heart.

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APPENDIX

Abbreviations used in this article are: ACE, angiotensin converting enzyme; Ad.CMV-cHK, adenovirus harboring the human tissue kallikrein gene; Ad.CMV-LacZ, adenovirus harboring the LacZ gene; BGH, bovine growth hormone; cAMP, 3′,5′-cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; CMV, cytomegalovirus; Dahl-SS, Dahl salt-sensitive rats; Dahl-SR, Dahl salt-resistant rats; GFR, glomerular filtration rate; NOx, nitrite/nitrate; PAH, para-aminohippuric acid; RPF, renal plasma flow; RT-PCR, reverse transcription-polymerase chain reaction.

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